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"THE PENETRATION AND MIGRATION OF SERRATIA MARCESCENS
IN BEREASANDSTONE"

BY

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A THESIS

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The undersigned hereby certify that they have read,
and recommend to the Faculty of Graduate Studies for
acceptance, a thesis entitled, "The Penetration and
Migration of Serratia marcescens in Berea Sandstone",
submitted by Ronald G.L. McCready, B.Sc., in partial
fulfillment of the requirements for the degree of
Master of Science in Microbiology.

ABSTRACT

The purpose of this project was to develop a technique for studying the migration and penetration of microorganisms in solid porous materials. S. marcescens was chosen as the test organism for these experiments as it is the approximate size of the various organisms which have been isolated from drilling muds and injection waters; also its vivid red pigment is useful in detecting the organism.

The organisms were labelled with radioactive phosphorus to facilitate tracing their rate of migration. The label has been shown to be stable. The distribution of the isotope in the cellular fractions of the organism has been determined.

Concentrated slurries of labelled S. marcescens were allowed to pass through Berea Sandstone cores. The migration of the organism was traced using a Geiger-Muller tube. At the end of each experiment the cores were split in two, one half was then coated with nutrient medium and incubated. The pigmented colonies of the test organism which developed clearly show the distribution of the organism within the core. The other half of the core was autoclaved and used to prepare an autoradiogram. Good correlation was obtained between colony development and distribution of P^{32} for each core.

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INTRODUCTION

The first reported work concerning the penetration and migration of bacteria through capillary spaces is that of Woodhead and Wood⁴² in 1894. They studied the penetration and migration of bacteria into and through bacterial filters which were available at the time. In 1924, Warren and Mudd³⁹ separated motile from non-motile organisms by passing the culture through a U tube containing 60/120 quartz sand. Separation was possible as the motile organisms traversed the filter medium more rapidly than the non-motile organisms.

In 1943, Merkt²⁰, working with sandstone and limestone cores, studied the plugging effect of four groups of micro-organisms isolated from injection waters; these were (1) iron oxidizing bacteria, (2) sulfur oxidizing bacteria, (3) sulfate reducing bacteria, and (4) algae. Work along similar lines was carried out by Plummer, Merkt, Power, Sawin and Tapp²⁶ in 1944, and by Plummer and Walling²⁷ in 1946. Recently Fekete⁷ and Raleigh²⁸ have studied the effect of B. subtilis in sandstone cores.

All previous studies on plugging have been carried out by passing the injection fluid through the cores under pressure. In the present work penetration and migration was studied under atmospheric pressure only. Woodhead and Wood⁴³ have shown that penetration and the rate of migration is increased when hydrostatic pressure is increased.

The palaeo-ecology of fossil bacteria is largely unstudied³³.

Bacteria are among the earliest micro-fossils recognized and are believed to date from the Proterozoic era. (Ellis ⁶, Grunner ⁹). The evidence supporting this theory is based entirely on microscopic examination of geological specimens or chemical extracts of such specimens ¹⁶.

In all previous reports it has been assumed that the organisms were trapped in the prehistoric formation at the time it was being formed. However, if the microorganisms can penetrate and migrate into porous formations, this assumption may be completely unfounded for the organisms could have entered the formation at some later date.

The purpose of the present study is to develop a technique for studying the penetration and migration of a motile organism into and through porous materials. The organisms were labelled with radio-active phosphorus in order to follow their migration through the porous material, without disturbing the physical or chemical character of the material. To our knowledge there have been no previous studies of this kind.

For the sake of clarity and coherence this thesis is divided into two main parts; Part 1 will deal with the problems associated with labelling of the organism, the distribution of the labelled element in the organism and evidence as to the stability of the labelled element within the organism. Part 11 will deal with studies on the penetration and migration of the labelled organisms through Berea Sandstone cores.

PART 1

MATERIALS AND METHODS

The organism used in these experiments was Serratia marcescens Bizio, ATCC (American Type Culture Collection) #274 or NCTC (National Collection of Type Cultures) #1377.

Cultivation

The cells were grown in three hundred millilitre aliquots of Bacto Nutrient Broth, with and without added glucose, containing varying amounts of radiophosphorus, and adjusted to a final pH of 7.0. The radiophosphorus ($\text{H}_3\text{P}^{32}\text{O}_4$ in dilute HCl) was obtained from Atomic Energy of Canada Limited, Chalk River, Ontario. Three one millilitre aliquots of the medium were taken before inoculation and dried on aluminum planchets for counting. The medium was then inoculated with one loopful of a forty-eight hour culture of cells grown on Nutrient Agar Plates. The cultures were incubated at various temperatures, but in all experiments they were aerated by shaking on a Model #10, New Brunswick Rotary Shaker at 350-400 oscillations per minute for 24 hours. After incubation, the viable cell counts were determined by plating out dilutions of one millilitre of the culture on Nutrient Agar Plates containing one percent glucose. Simultaneously the cells were harvested using a Servall SS-1 Centrifuge at 9,5000 r.p.m. for ten minutes, resuspended in thirty millilitres of distilled water and washed three successive times to remove any loosely bound P^{32} . The total radioactivity of the labelled cells was determined by

resuspending the cells in a known volume of distilled water and counting one millilitre of the suspension which had been dried on an aluminum planchet.

Counting and Calculation

In all experiments the counts were determined using a Model 186 A Decade Scaler at an operating voltage of 500 volts and a sensitivity setting of 250 with a Model D-39 Geiger-Muller Tube which has a 1.4 mg./cm^2 mica window; both of which were purchased from the Nuclear of Chicago Corporation. Percent uptake of the P^{32} was determined by dividing the total cell count by the total count of the original medium times one hundred per cent. It should be noted that in experiments of less than thirty-six hours duration; all samples for counting were held until completion of the experiment so that all counts could be made at the same time, thereby avoiding the use of the decay factor and avoiding possible errors. In the longer experiments the counts were made on the various fractions as they were isolated and all counts were calculated back to time zero for that experiment.

Phosphorus Retention

Four twenty-five gram samples of powdered sandstone were respectively eluted for twenty-four hours with 100 millilitre aliquots of normal saline, $1/10 \text{ M. trishydroxymethyl amino methane}$ (Tris Buffer), disodium phosphate buffer and distilled water. Two to three grams, wet weight of labelled cells were placed in a dialysis membrane which had been washed once with ninety-five percent ethanol and three times with demineralized water before

sterilization. The dialysis bag was then suspended in one hundred millilitres of the various test solutions containing the soluble fraction from the twenty-five grams of sandstone. The volume of the external phase was kept constant throughout the course of the experiment, and it was also kept in constant motion by the use of a magnetic mixer. One millilitre aliquots were withdrawn from the external phase at various time intervals, in duplicate, counted and the results averaged.

Cell Fractionation

A. Combination and Modification of the Schmidt-Thannhauser³² and Stuy³⁶ Techniques.

Ten millilitres of cold five percent trichloroacetic acid were added to the washed cells in a fifty millilitre polypropylene centrifuge tube which was held at 4°C. for ten to fifteen minutes to facilitate the removal of the organic acid soluble fraction. The cells were then centrifuged and the supernatant decanted, one millilitre of which was dried and counted. The cell residue was washed twice with five millilitre aliquots of ninety-five percent ethanol and again one millilitre of the decanted supernatant was taken and dried on an aluminum planchet for counting. The total radioactivity was then calculated for the total volume of the supernatant and the counts were taken to represent the lipid fraction.

Four millilitres of 1 N. potassium hydroxide were then added to the cell residue and incubated at 45° C. for seventeen hours to hydrolyze the ribonucleic acid (RNA) fraction. The hydrolysate was then neutralized to pH 7.0 with perchloric acid and centrifuged.

Again the supernatant was decanted and one millilitre aliquot was dried and counted. To the residue containing the deoxyribonucleic acid (DNA), five millilitres of five percent trichloroacetic acid were added, and the mixture was heated to 90°C. on a water bath for fifteen minutes and allowed to cool to room temperature (25-27°C.) and centrifuged. One millilitre of the supernatant was taken, dried and counted.

B. Phenol Extraction

Cellular studies were also carried out using the technique outlined by Blobel ⁴. The cells were harvested and washed four times in 0.14 M. sodium chloride and 0.015 M. sodium citrate. The cells were then suspended in ten millilitres of cold five percent trichloroacetic acid and the acid soluble fraction removed. The residue was then extracted with two, five millilitre, aliquots of ninety-five percent ethanol to remove the lipids. The DNA fraction was extracted by suspending the residue in ten millilitres of 2 M. sodium citrate and water saturated phenol (approximately 88% phenol) mixed 1:1 at a pH of 7.4. The suspension was kept at 4°C. overnight and then centrifuged at 9,500 r.p.m. for twenty minutes. Treatment of the aqueous phase with an equal volume of liquified phenol was repeated twice at pH 7.4, with gentle shaking. After the residual phenol had been removed by extensive dialysis against 0.14 M. sodium chloride and 0.015 M. sodium citrate, DNA was precipitated from the aqueous phase by layering upon it an equal volume of 2-ethoxyethanol. The layers were slowly mixed and the fibrous precipitate was collected on a stirring rod and

dissolved in 0.14 M. sodium chloride and 0.015 M. sodium citrate overnight. Insoluble residues were subsequently removed by centrifugation. The total phenolic fraction which had been retained was counted and the total count taken as RNA.

C. Extraction of the Pigment (Prodigiosin)

As there was a possibility of the Prodigiosin containing a fraction of the label; the pigment itself was extracted using a modification of the technique outlined by Williams, Green and Rappoport ⁴¹. S. marcescens was grown in Nutrient Broth with one percent glucose and 10 microcuries ($\mu\text{c.}$) of P^{32} at 30° C. for twenty-four hours, and then allowed to stand at room temperature for an additional twenty-four hours for maximal pigmentation. The cells were centrifuged at 9,500 r.p.m. for ten minutes and then washed three consecutive times with distilled water. The washed cells were then placed in an asbestos Soxhlet cup and one hundred and twenty-five millilitres of acetone and petroleum ether in a ratio of 2:1 were added. The extraction was carried out for four hours in a Soxhlet Apparatus. The extract was then concentrated in a Flash Evaporator. The total pigment was then dried and counted.

D. Phosphoprotein Determination

The isolation of the phosphoprotein fraction was carried out by using a modification of the technique put forth by Delory ⁵. After the extraction of the DNA by the Schmidt-Thannhauser technique the contents of the centrifuge tube were neutralized to phenolphthalien by the dropwise addition of concentrated ammonium hydroxide. An extra 0.2 millilitres of the same solution was then added. After

the addition of one millilitre of 2.5% calcium chloride solution and one millilitre of a 0.5% suspension of "light" magnesium carbonate in water, the tube was shaken and allowed to stand for thirty minutes. It was then shaken again and centrifuged. The supernatant was decanted and a count taken of the residue which had been dried on a planchet.

E. Total Phosphorus Determination

For specific activity determinations the total phosphorus of each isolated cell fraction was determined using a modified Kuttner and Lichenstein¹² procedure. Each fraction was placed in a Kjeldahl Flask to which was added one millilitre of 10 N. sulfuric acid. The mixture was then heated to charring. When white fumes appeared the flame was reduced to a minimum and thirty percent hydrogen peroxide was added drop by drop until the solution remained clear with gentle heating. The flasks were then heated to remove the excess hydrogen peroxide and a small amount of water was added to complete the removal. The solutions were cooled to room temperature and demineralized water was added to give a total volume of eight millilitres. To this one millilitre of molybdate reagent and one millilitre of dilute stannous chloride were added. The total phosphorus was then determined colorimetrically at 660 mμ using the Beckman D.U. Spectrophotometer.

RESULTS

Radiophosphorus Uptake

As the radiophosphorus was being used primarily as a tracing agent it was not necessary to incorporate large amounts into the

organisms. Fuerst and Stent⁸ have shown that if the specific activity ($S/A = \mu\text{g. P}^{32} \text{ per } \mu\text{g. total phosphorus}$) of the medium is too high, the lethal effect of the radiation is also very high. A series of experiments were done to determine the amount of radiophosphorus required to give a detectable label with minimal lethal effect. The results of these experiments are shown in TABLE 1.

TABLE 1

THE EFFECT OF RADIOPHOSPHORUS CONCENTRATION ON CELL VIABILITY
AND PHOSPHORUS UPTAKE

Cultures grown in Nutrient Broth at pH 7.0, incubated
at 37°C for twenty-four hours

$\mu\text{c. P}^{32}/$ 300 mls.	Activity of cells (c/m)	Total medium counts/minute	% of P^{32} in cells	Viable cells per ml.
200	195,636	2,740,000	7.14	5.25×10^8
100	160,753	1,637,000	9.82	9.10×10^8
50	70,761	702,000	10.08	9.20×10^8
10	43,097	285,600	15.09	3.08×10^9
10	45,686	280,800	16.27	3.27×10^9
Control	0	0	0	3.52×10^9

The Effect of Glucose and Temperature on Radiophosphorus Uptake

In preliminary experiments we found that phosphorus uptake was stimulated by glucose and that pigmentation of the S. marcescens was increased by incubating the culture at 30° C. rather than at 37° C. Experiments were done to determine the level of glucose which would have a maximal stimulatory effect on phosphorus uptake. Once this was determined, a comparison of the radiophosphorus uptake was made at 30° C. and 37° C. The results of these experiments are outlined in TABLES II and III.

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TABLE 11

THE EFFECT OF VARIOUS CONCENTRATIONS OF GLUCOSE ON THE UPTAKE
OF RADIOPHOSPHORUS BY S. MARCESCENS

Experiment	Percentage uptake of the available P^{32} when glucose concentration is:			
	0% w/v	1% w/v	1.5% w/v	2.0% w/v
1	15.09	23.40	23.60	23.20
2	16.27	19.40	19.00	19.10
3		19.70	19.00	19.10
Average	15.68	20.80	20.60	20.20

TABLE 111

COMPARISON OF THE UPTAKE OF RADIOPHOSPHORUS BY S. MARCESCENS GROWN
IN NUTRIENT BROTH WITH 1% GLUCOSE ADDED AT 30° C. AND AT 37° C.

Experiment	Percentage uptake of the available P^{32} at:	
	30° C.	37° C.
1	32.80	23.40
2	32.02	19.40
3	32.87	19.70
Average	32.56	20.80

In all subsequent experiments the organisms were grown in Nutrient Broth with 1% glucose and were labelled with 10 microcuries (μc) of P^{32} per 300 mls. of medium. The pH was adjusted to 7.0 and incubation was carried out at 30° C. for twenty-four hours on the oscillating shaker.

Retention of the Label

As subsequent experiments involving the passage of the labelled organisms through the sandstone cores were to be conducted over fairly

long periods of time, it was necessary to determine the stability of the label. In addition, commonly used buffers were tested to determine which fluid would be most satisfactory as a vehicle for the organisms in the migration studies. One hundred millilitres of the fluids to be tested were added, individually, to 25 grams of powdered Berea Sandstone and placed on a magnetic mixer for twenty-four hours. Thus, soluble material from the core would be present in the solution to be used for the dialysis of the labelled cells. TABLE IV lists the fluids tested as well as their original pH and the pH after the elution of the soluble material from the sandstone.

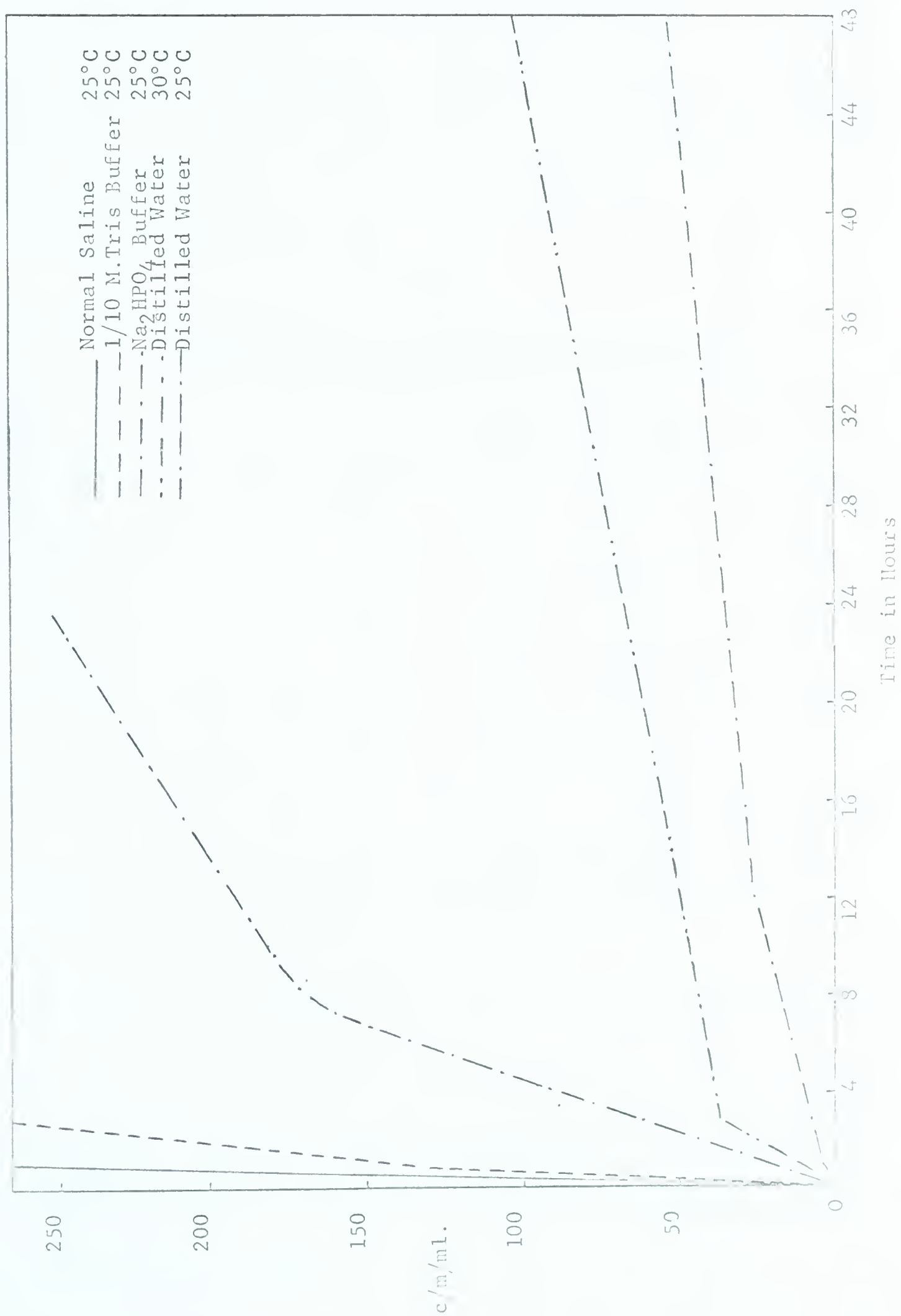
TABLE IV
SOLUTIONS USED IN RADIOPHOSPHORUS RETENTION STUDIES

Solution	Original pH	Final pH
Normal Saline	7.2	7.2
Disodium Phosphate Buffer	7.38	7.5
1/10 M. Tris Buffer	7.2	7.3
Distilled Water	6.8	7.6

The results of the phosphorus retention studies are given in Figure 1. Each curve is the average of three experiments. After distilled water had been found to be the best vehicle, a series of experiments were carried out to give an indication as to the effect of temperature on radiophosphorus retention.

FIGURE I

RADIOPHOSPHORUS RETENTION BY S. NARCESCENS DURING DIALYSIS WITH VARIOUS FLUIDS



Distribution of P³² in S. marcescens

Since Mulvey ²⁵, in 1962, reported a rapid loss of P³² from Trypanosoma equiperdum (57% loss in the first hour after harvesting the organisms from the medium) a distribution study was done to determine why S. marcescens retained the label over fairly long periods of time in our experiments. The results of these studies are outlined below. The results are given as a percentage of the total P³² in the cell sample.

TABLE V

DISTRIBUTION OF P³² IN S. MARCESCENS
DETERMINED BY THE SCHMIDT-THANNHAUSER TECHNIQUE

Fraction	Experiment Number				
	1	2	3	4	5 *
Acid Soluble	17.76	17.12	22.43	16.61	17.52
Lipid	6.03	7.36	7.44	12.09	9.74
Ribonucleic Acid	62.70	60.72	55.90	57.75	57.26
Deoxyribonucleic Acid	1.85	8.10	2.82	8.06	4.39

* Extraction carried out as per Blobel ⁵.

As pigmentation occurs with S. marcescens after twenty-four hours, and as our penetration studies often were conducted for extended periods of time, the amount of P³² in the pigment was determined. The average figure for three determinations was 5.57% of the total cellular P³².

In 1961, Guelin and Lepine ¹⁰ reported that approximately 20% of the P³² taken up by Shigella paradysenteriae was found in the phospho-protein fraction. On analysis of S. marcescens no phospho-protein could be found.

Phosphorus Uptake and Distribution During Incubation

S. marcescens was grown in Nutrient Broth containing 1% glucose at 30°C. for 24 hours. The cells were harvested, washed once with distilled water and transferred to 1100 cc. of growth medium containing 10 µc. of P³². The culture was then incubated at 30°C. for the course of the experiment, and at various time intervals 30 millilitre aliquots were taken, the cells harvested, and the biochemical analysis was carried out.

TABLE VI

UPTAKE AND DISTRIBUTION OF P³² IN S. MARCESCENS DURING INCUBATION

Time of Sampling	<u>Counts per minute in excess of background</u>			
	Acid Soluble	Lipid	R.N.A.	D.N.A.
5 minutes	929	237	0	0
10 minutes	1,212	237	149	0
15 minutes	1,287	135	134	0
30 minutes	1,187	143	340	0
45 minutes	2,440	216	888	255
60 minutes	2,386	143	1,395	384
90 minutes	1,885	227	2,226	328
2 hours	2,484	508	5,974	807
3 hours	3,461	370	7,454	900
4 hours	3,836	728	10,462	905
8 hours	5,432	2,089	19,318	2,046
14 hours	5,258	1,664	19,902	2,330
16 hours	4,333	2,033	19,950	1,864
20 hours	4,564	1,810	21,052	2,599
24 hours	4,589	1,930	20,896	2,583

At the end of twenty-four hours incubation with 10µc. of P³², the cells had assimilated 34.46% of the available isotope.

The percentage distribution of the assimilated radio-isotope is outlined below in Figure 11.

FIGURE II
THE DISTRIBUTION OF P^{32} ASSIMILATED BY S. MARCESCENS DURING INCUBATION

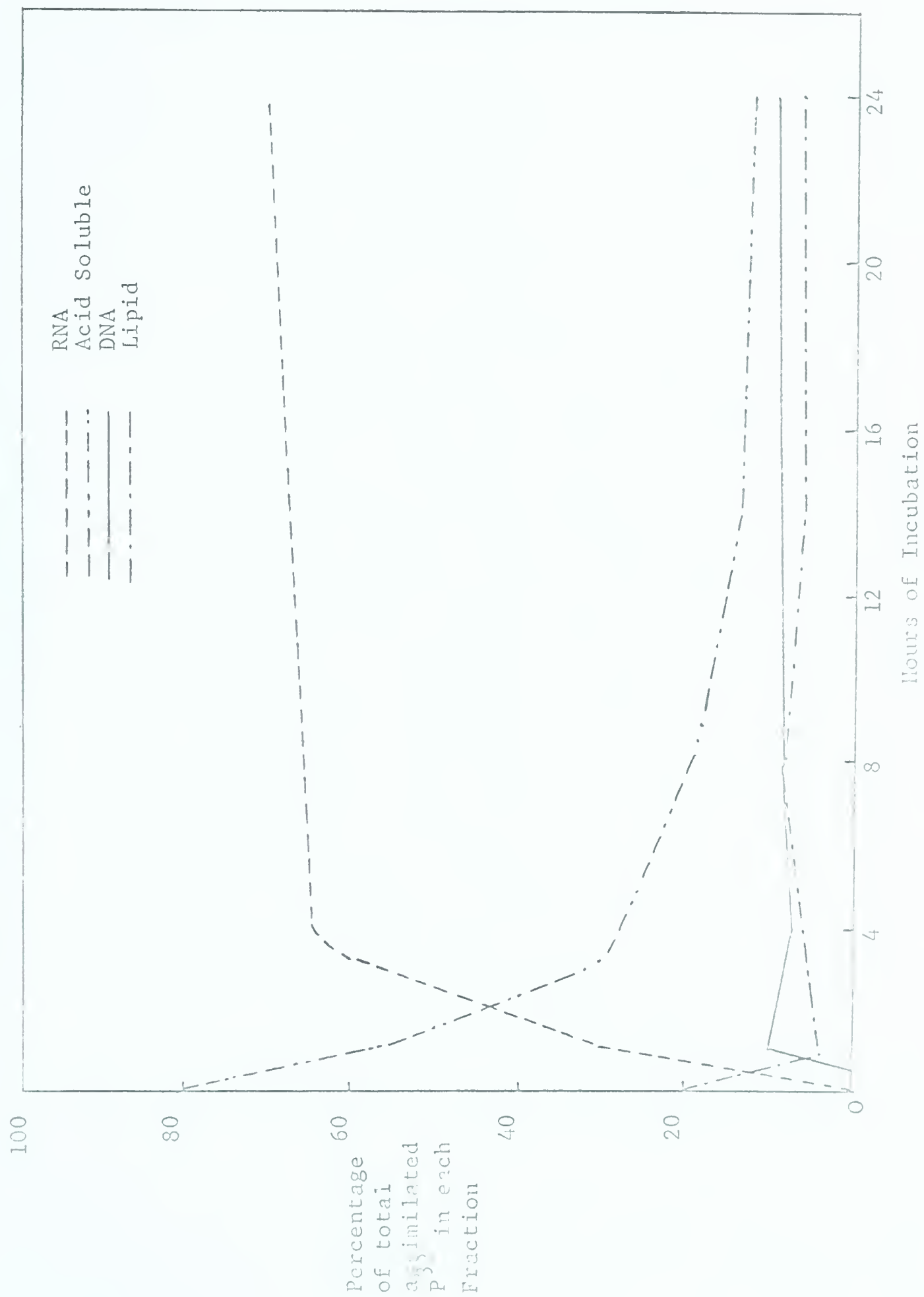
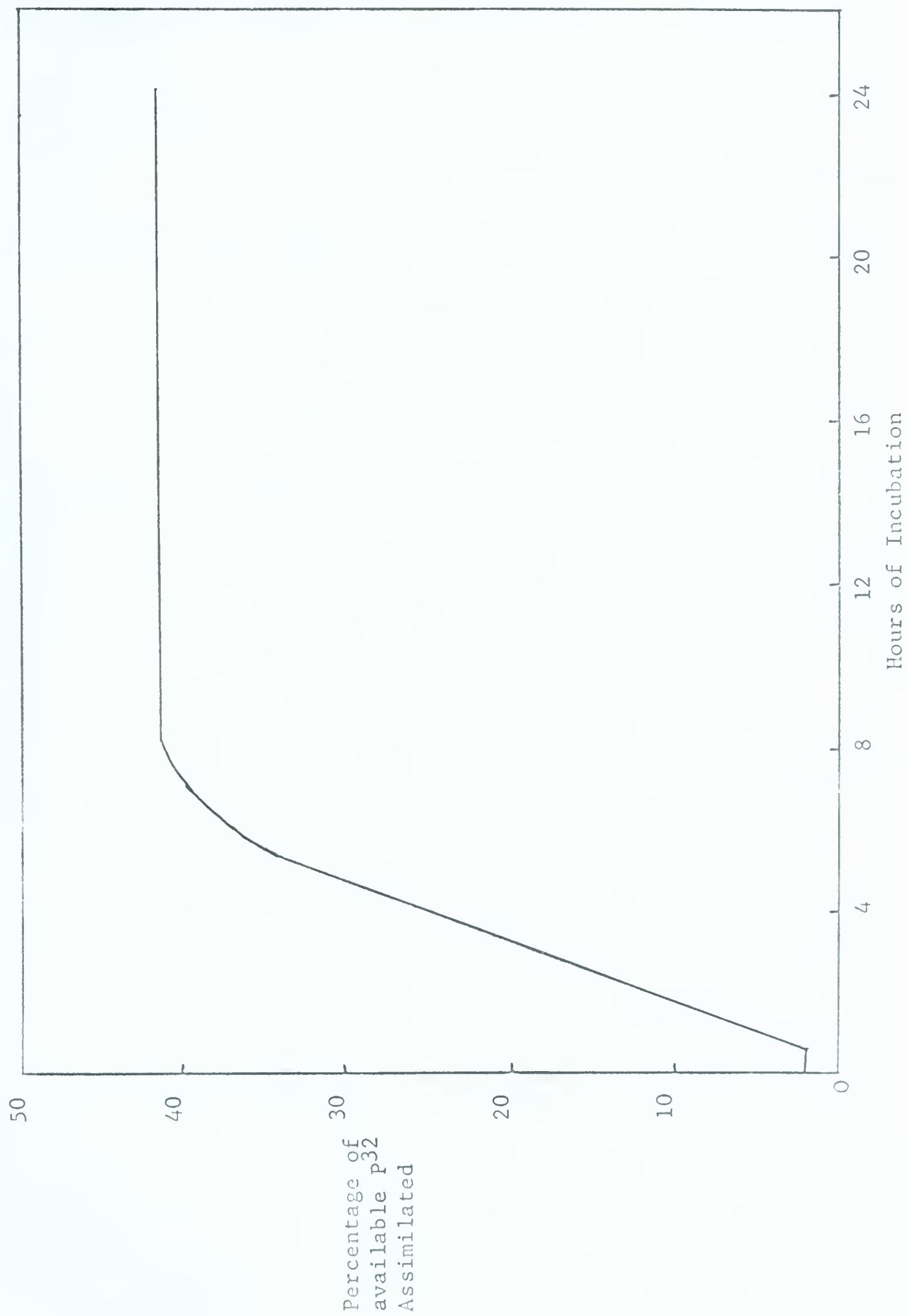


FIGURE III
RATE OF ASSIMILATION OF P^{32} BY S. MARCESCENS



Specific Activity of the Cellular Fractions

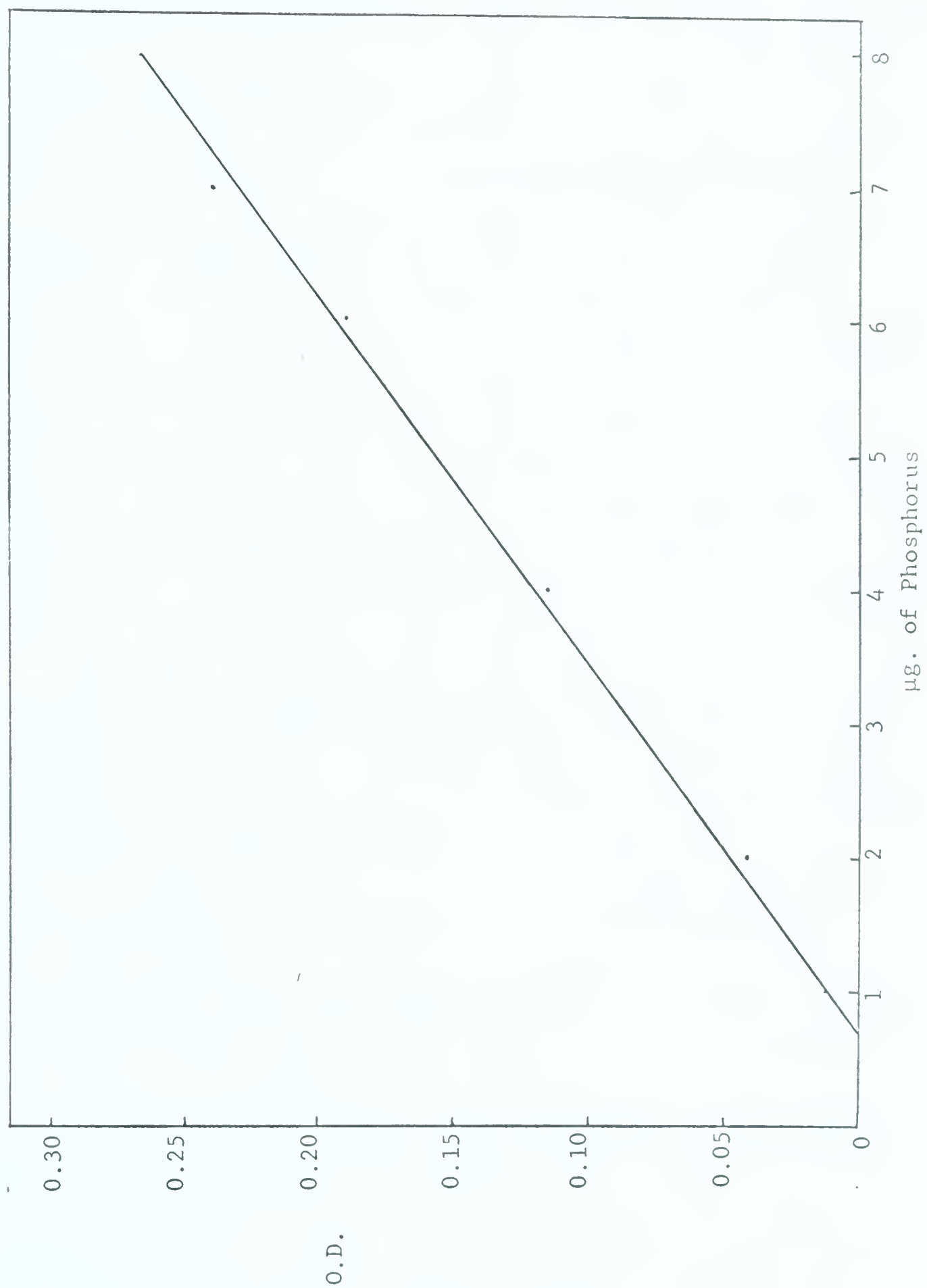
The cellular fractions were digested using a Micro Kjeldahl Apparatus and the procedure of Kuttner and Lichenstein¹². The standard phosphate solution was prepared by adding 0.4394 grams of dried KH_2PO_4 to one litre of demineralized water. One millilitre of this solution was equivalent to 0.1 mg. of phosphorus. The results of this experiment are outlined in TABLE VII.

TABLE VII

THE SPECIFIC ACTIVITY OF THE CELLULAR FRACTIONS
OF P^{32} LABELLED S. MARCESCENS

Fraction	O.D. of a 1:1000 dilution	mg. P	counts/min.	Specific Activity
Acid Soluble	0.080	2.94	60,039	20,044 c/m/mg. P
Lipid	0.036	1.48	20,638	10,885 c/m/mg. P
R.N.A.	0.217	6.67	202,300	33,390 c/m/mg. P
D.N.A.	0.057	2.32	5,618	2,421 c/m/mg. P

FIGURE IV
STANDARD GRAPH FOR PHOSPHORUS DETERMINATION



DISCUSSION

Labelling of the Organisms

In tracer studies, the object in labelling of biological materials is to obtain sufficient label for counting while causing no deleterious effect to the material. Fuerst and Stent⁸ state that the rate of P^{32} assimilation is proportional to the specific activity of the growth medium. Hence the bacteria in a high specific activity medium incorporate initially many more atoms of P^{32} into their phosphorylated constituents per unit time than those in a low specific activity medium. It therefore follows that if the uptake is initially high, the death rate will also be very high early in the experiment; thereby reducing the initial population and thus reducing the total number of cells produced in the twenty-four hour incubation period.

From TABLE 1 it can be seen that cells grown under standard conditions of pH, temperature, medium and aeration, with varying concentrations of P^{32} in the medium show a great variance in the percentage of isotope assimilated as well as in viable count. It can be seen that the viable count is inversely proportional to the concentration of P^{32} added. It is evident that a concentration of 10 μ c. of P^{32} per three hundred millilitres of medium has no significant lethal effect on the organisms.

Fuerst and Stent⁸, McFall, Pardee and Stent¹⁸, and McFall and Stent¹⁹ have studied the death rate of E. coli due to incorporated P^{32} . They have found that death is due to inactivation of the DNA by the radiation from the isotope. Schmidt³¹, has found that only 45% of the cells of E. coli exposed to a

concentration of 50 μ c. of P^{32} in 55 millilitres of medium survive after seventy-two to ninety-six hours; and only 22.9% survive after two hundred and eighty-eight hours.

In 1955, Stent and Fuerst ³⁵, postulated that the lethal effect of P^{32} in the DNA was due to the transmutation of P^{32} to S^{32} on decay. This theory is supported by the work of Van Dyke ³⁷, and Apelgot and Latarjet ².

Lea, Haines and Bretscher ¹⁴, have calculated that 4×10^3 Roentgens of beta (β) emission are required per cell to kill E. coli. The emission rate for the amount of isotope used in our experiments (10 μ c. P^{32} /300 mls. of medium) is less than one Roentgen per second. As this emission is distributed throughout the total volume of the medium it will have no lethal effect.

It was determined experimentally that almost all of the loosely bound or surface adherant P^{32} was removed from the harvested cells by three successive washings with distilled water. However, it is interesting to note that even after twelve to fifteen successive washings minute amounts of P^{32} could be detected in the supernatant after centrifugation.

Effect of Glucose on P^{32} Assimilation

The results presented in TABLE II indicate that glucose stimulated phosphate uptake by approximately 35%. The stimulation is believed due to the role of phosphate in the metabolism of glucose. Rothstein ³⁰ suggests that phosphate is esterified in the cell membrane and that it is then passed on to the metabolic pools. Once the phosphate is in the metabolic pool it is available for esterification with glucose, via

adenosine triphosphate, producing glucose-6-phosphate which is then metabolized via the various pathways.

In addition the results shown in TABLE II reveal that stimulation of phosphorus uptake is not increased by increasing the concentration of glucose beyond the optimum level of 1% w/v. In terms of enzyme kinetics, once the concentration of glucose is sufficient for enzyme saturation, the metabolic activity of the enzyme will be maximal.

Effect of Temperature on P^{32} Assimilation

From our preliminary studies it is known that pigmentation in S. marcescens is stimulated to a greater extent by incubating the organisms at 30° C. rather than at 37° C. It is evident from the results shown in TABLE III that a 50% increase in P^{32} assimilation is obtained by incubating the cells at 30° C.

Retention of the Label

Figure 1 shows that the loss of P^{32} from the cells is very rapid when the cells are dialysed with normal saline, dibasic sodium phosphate buffer or 1/10 M. Tris Buffer at 25° C. The loss of P^{32} is much less when distilled water is used, but loss increases with a slight elevation in temperature.

It is interesting to note that the loss of the label appears to coincide with the pH of the test solution. The lower the pH, the more rapid the loss of the label. Another factor which may enter into the loss of P^{32} , is the presence of the sodium ion. Rothstein³⁰ has shown a correlation between phosphate transport across the cell membrane and the transport of the sodium ion or potassium ions. Therefore it appears that the presence of the sodium ion may stimulate the transfer of the phosphate ions from the cells into the medium. Another mechanism for phosphate release which applies in the case of

the disodium phosphate buffer is the exchange of the labelled phosphate from the cell with the non-labelled phosphate of the buffer.

Distribution of P^{32} in *S. marcescens*

Results given in TABLE V show that 60 to 65% of the P^{32} assimilated by the cell is in the nucleic acids.

In Experiment #3 the high acid soluble count is believed due to the use of warm trichloroacetic acid which may have caused a partial breakdown of the DNA thereby increasing the level of activity in the acid soluble fraction.

Blobel's procedure was discarded in favor of the Schmidt-Thannhauser procedure as the latter was shorter and the percentage recovery of the isotope was equal to, and often better than with the former.

Roberts, Abelson, Cowie, Bolton and Britton²⁹ have carried out isotope assimilation experiments with *E. coli*. As *S. marcescens* and *E. coli* are similar in cellular morphology, size and Gram reaction a comparison of their isotope uptake seems feasible. Below is a comparison of the distribution of P^{32} in *E. coli* as obtained by Roberts et al.²⁹ with the average results of our Schmidt-Thannhauser extraction with *S. marcescens*.

TABLE VIIA

COMPARISON OF THE DISTRIBUTION OF P^{32} IN
E. COLI AND *S. MARCESCENS*

<u>Fraction</u>	<u><i>E. coli</i></u> ²⁹	<u><i>S. marcescens</i></u>
Acid Soluble	17.60	18.38
Lipid	13.37	8.20
Nucleic Acids	62.10	64.45

The retention of the P^{32} by S. marcescens may be due to the accumulation of the majority of the isotope in the nucleic acids. Mandelstam¹⁷ states that evidence from isotope labelling experiments indicates that DNA is very stable; and that RNA degradation begins only after depletion of specific amino acids, nitrogen, pyrimidine, magnesium or phosphate.

Levy, Skutch and Shade¹⁵ found the turnover time for RNA-phosphorus was 12.2 hours and for DNA-phosphorus, 32.9 hours in Proteus vulgaris. Therefore, as the nucleic acids have relatively long turnover times, this may be the main factor in the retention of the label in our experiments.

The contention of Guelin and Lepine¹⁰ that 20% of the assimilated P^{32} accumulates in the phosphoprotein fraction of Shigella paradysenteriae does not seem reasonable. Using a modified Schmidt-Thannhauser Technique, Roberts et al.²⁹ could not isolate phosphoprotein from E. coli; and using our extraction procedure with S. marcescens no phosphoprotein could be detected. The three organisms mentioned above are all Gram negative in their staining reaction; Mitchell and Moyle²² state that phosphoproteins are absent in all Gram negative organisms, but present in Gram positive organisms, and they are believed to be a constituent of the cell envelope.

Our result showing 5.57% of the total cellular radiophosphorus present in the pigment is considered invalid since hot acetone was used for the extraction and this solvent would extract not only the pigment from the cell but also the lipid components. (Hubbard and Rimington¹¹).

Phosphorus Uptake and Distribution during Incubation

Results presented in Figure VI show that incorporation of the isotope begins almost immediately in the acid soluble and lipid fractions of the cells. There appears to be a slight delay in the incorporation of the isotope into the RNA and quite a prolonged delay in the incorporation into the DNA. The results obtained for the nucleic acids agree with the work of Morse and Carter²³ using E. coli, strains B and B/r, and with the results of Wade and Lovett³⁸.

It is realized that the results obtained in this experiment may not be a true representation of the uptake and distribution of P^{32} during cultivation since the cells from a twenty-four hour culture were used as the inoculum. This procedure was necessary in order that a sufficient number of cells would be present in the aliquots taken at the various time intervals for the biochemical analysis.

The curve in Figure III showing the uptake of P^{32} during incubation closely resembles the normal growth curve for bacteria³⁴. This is to be expected since Labaw, Mosley and Wyckoff¹³ have stated that the uptake of an isotope and multiplication continued in E. coli until the onset of logarithmic decay.

Specific Activity of the Cellular Fractions

It can be seen (TABLE VII) that RNA has the highest specific activity and this is believed to be due to the continuous synthesis of RNA during growth and multiplication of the cells. A small proportion of the RNA, called messenger RNA, is destroyed during protein synthesis, if the cell is to maintain a constant level of RNA under these conditions RNA must be continuously synthesized

during the growth of the cells. RNA synthesis is occurring in the presence of radiophosphorus, and since RNA is the largest single constituent of the cell protoplasm, it is logical that it should have the highest specific activity. DNA on the other hand has a low turnover time and is synthesized only during cell division and therefore it has a low specific activity.

Mitchell and Moyle²¹ state that there is no direct or indirect transfer of phosphorus from RNA to DNA or vice versa. They also state the phospho-lipid shows a rapid turnover during respiration and growth, thereby explaining the relatively high specific activity.

PART II

PENETRATION AND MIGRATION STUDY IN BEREASANDSTONE

MATERIALS AND METHODS

The cores used in these studies were of Berea Sandstone which had been cut parallel to the bedding plane so that the grain of the core runs longitudinally. The general chemical composition of Berea Sandstone is shown below in TABLE VIII.

TABLE VIII

THE CHEMICAL COMPOSITION OF BEREASANDSTONE (Weidman⁴⁰)

<u>Component</u>	<u>Percentage</u>
Silicon oxide	93.47
Aluminum oxide	2.77
Ferric oxide	0.01
Ferrous oxide	0.81
Ferrous Sulfide	0.01
Magnesium Oxide	0.03
Calcium Oxide	0.44
Sodium Oxide	0.29
Potassium Oxide	0.51
Water	0.02
Deuterium Oxide	0.75
Carbon Dioxide	0.74
Titanium Oxide	0.25
Phosphorus Pentoxide	0.03
Sulfite	0.01
Manganese Oxide	0.04
Zirconium Oxide	0.01
Carbon, organic	0.01

It can be seen that the level of phosphorus is very low, thereby eliminating the possibility of phosphorus exchange between the core and the labelled organisms. Analysis of our core samples by emission spectrography indicated little or no phosphorus to be present. The individual particles of the cores range in size from one to 500 microns. The calculated pore size varies from one micron to 76 microns in diameter, with the majority of the pores being one to three microns in diameter. (See pore distribution curve in the Appendix)

In order to prevent the migration of the labelled organisms along the external perimeter, the cores were sealed with epoxy resin, in addition the epoxy seal acted as shielding, protecting the experimenter from excessive beta irradiation. In preliminary experiments it was found that a piece of one quarter inch thick epoxy resin reduced the level of radiation from a sample, emitting approximately 10,000 c/m., to a level slightly above background. It is believed that the epoxy resin absorbs the beta particles in a manner similar to beta absorption by lucite.

A quarter inch layer of epoxy resin around the perimeter of the core was obtained by using a two and one half inch, internal diameter, cardboard tube as a mold. The internal surface of the tubing was evenly coated with a silicon lubricant to facilitate removal of the mold once the epoxy resin had set. The core was centred within the tubing, and its position was maintained by placing four pieces of one quarter inch lucite rod equidistantly around the upper and lower ends of the core. The epoxy resin

was then prepared as follows; twenty-five parts of Celite 281, which acts as a filler, were added to 100 parts of Epon Resin 828 and a homogenous mixture was obtained by stirring. Twenty parts of Epon Curing Agent T-1 were then added with constant stirring and a homogenous mixture was obtained as quickly as possible. The resulting mixture was poured into the mold around the core; the resin began to harden within fifteen minutes. (See Appendix)

The resin was allowed to set for twenty-four hours. At this time, four series of ports were drilled through the epoxy along the longitudinal axis of the core at one inch intervals, each port being one quarter inch in diameter and each series being equidistantly spaced around the external circumference of the core. The four series of ports were marked 0° , 90° , 180° , 270° respectively to facilitate identification during the penetration and migration studies.

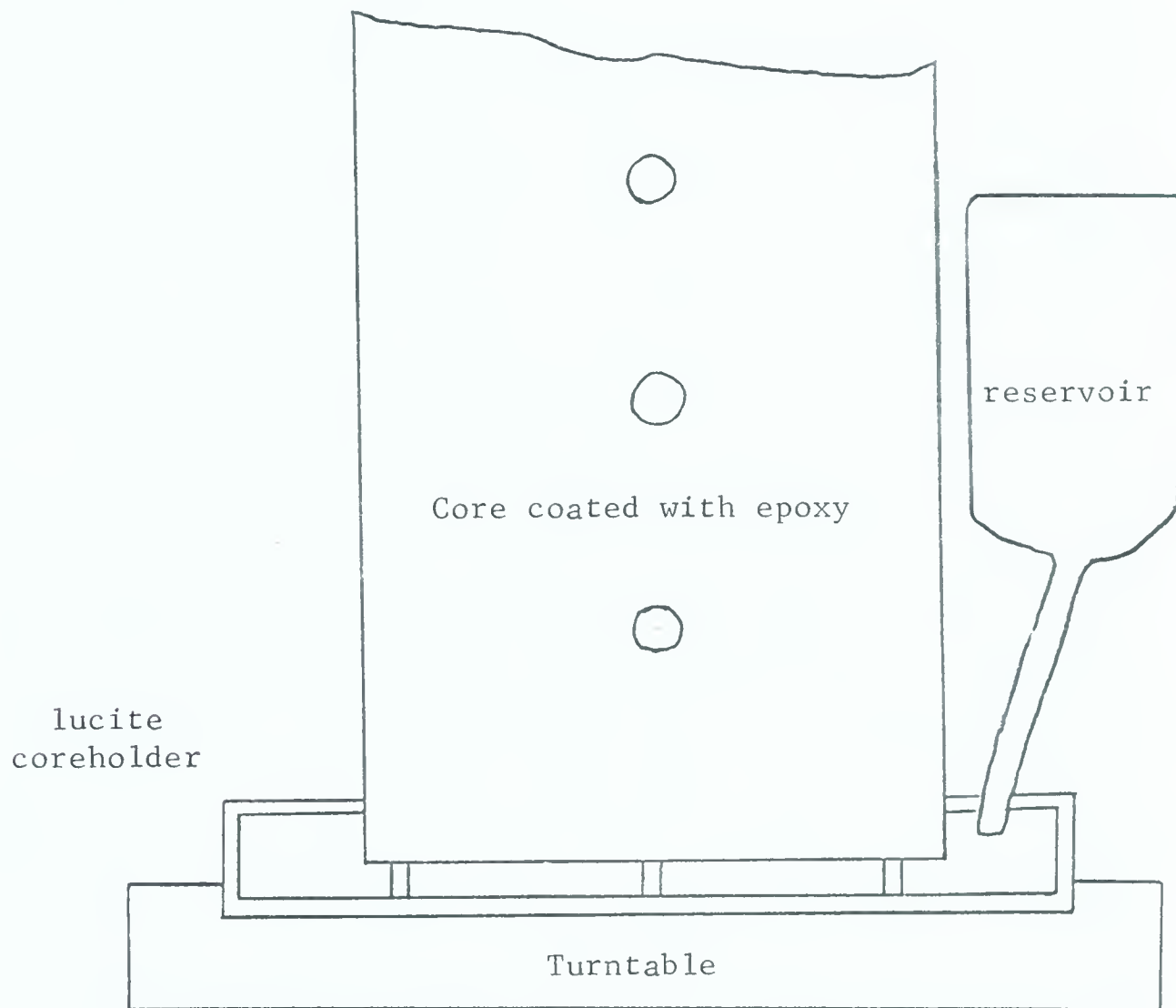
PLATE 1

A PREPARED CORE



FIGURE V

LUCITE CORE HOLDER WITH RESERVOIR



The Lucite core holder is placed on a turntable to facilitate maintenance of geometry while counting. Four small lucite pins support the core when it is immersed in the slurry.

After preparation the cores were subjected to a vacuum of 770 mm. of mercury for 24 hours at room temperature (25-27° C.) to facilitate drying. While the cores were being evacuated, a culture of P^{32} labelled S. marcescens was prepared as previously described. The cells were removed from the medium by centrifugation, washed three successive times with distilled water and the percentage assimilation of P^{32} was calculated. The cells were resuspended in distilled demineralized water to give a final volume of 126 millilitres. A one millilitre sample was taken for the plate count determination and the remaining 126 millilitres were placed in a sterile polyethylene reservoir which was attached to a lucite core holder.

The evacuated core was removed from the vacuum chamber and placed in the lucite container (see Figure V) and the slurry was added from the reservoir, which was designed to maintain a constant fluid level of slurry in the core holder, thereby preventing a disruption in the capillary action during the experiment. Suspension of the cells was maintained by bubbling sterile air through the slurry during the experiment. Counts were taken at each port, every four hours until the organisms could be detected at the top of the core by swabbing and subsequent plating on Nutrient Agar containing 1% glucose.

In preliminary experiments it was established that 0.05% of the activity from a P^{32} standard sample, emitting 10,000 c/m could be detected through a one half inch piece of Berea Sandstone. It is improbable that a sufficient concentration of labelled cells could accumulate at a given point to give this high an activity, therefore in the migration studies, counts taken at the core perimeter, are assumed to be

attributable to organisms that are within the first eighth to one quarter inch from the exterior. Unless there is a very high concentration of the labelled organisms at or near the centre of the core these organisms cannot be detected.

When evidence was obtained that migration of the organisms through the total length of the core had occurred, the core was split longitudinally by scoring with a carborundum blade, followed by cleaving with a piece of sterile strap iron and a hammer. (See Figure VI). The majority of the cores so treated split cleanly with minimal breakage. Cleaving was done as quickly and as carefully as possible to prevent contamination. One half of the core was coated, to a depth of one quarter inch, on the split surface with Nutrient Agar containing 1% glucose and incubated at 30° C. for 24 hours. The second half was placed in a sterile container and autoclaved; an autoradiogram was then prepared by covering the split surface with a thin plastic film (Saran Wrap) to prevent scratching of the x-ray film. The core was then placed, split surface down, on a x-ray film in a light-tight box. The box was set aside for four or five weeks, after which the film was processed and prints were prepared.

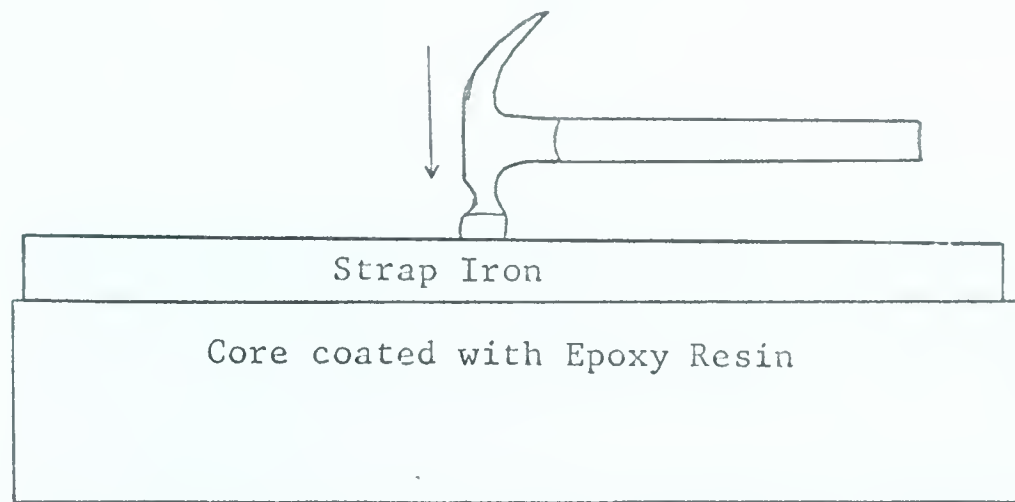
In all determinations, counts were taken for three minutes and then divided by three. Counts which were not at least 10 percent above the background count were reported as background; all other figures in the tables refer to c/m above the background count. For counts taken at the core ports, the Geiger-Muller Tube was

fitted with a plastic cap which had an eighth inch hole centrally located. This cap reduced the background count, and the hole acted as a guide in positioning the G-M tube while counting at the ports, thereby maintaining constant geometry for each count.

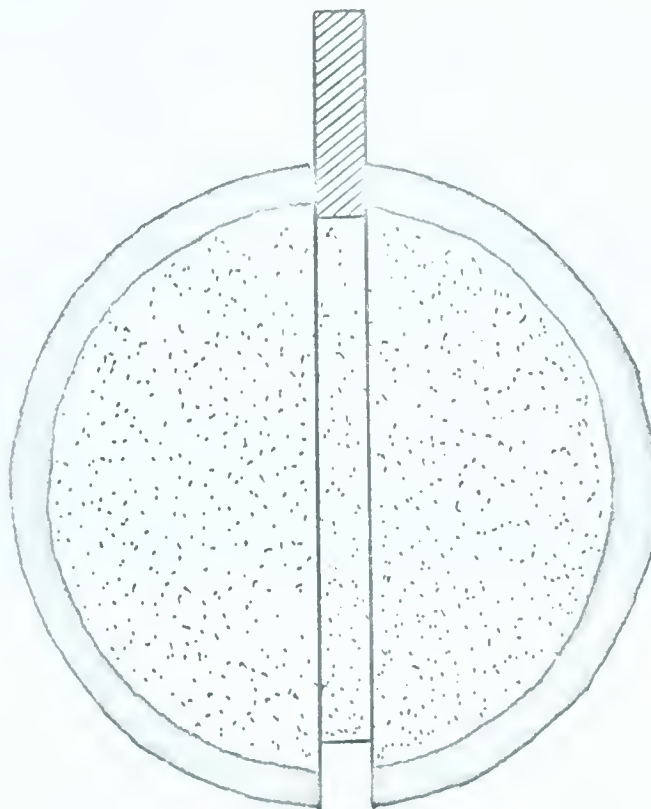
The G-M tube was held stationary at each core level, and the core, which was on a turntable, was turned 90° after each count to facilitate counting at the next port. (See Plate 11)

In this way, the geometry of the G-M tube was maintained at each level.

FIGURE VI
METHOD OF SPLITTING THE CORES



Side View



End View

PLATE II

APPARATUS FOR TRACING MIGRATION



RESULTS

Core #1 was exactly seven inches long. The slurry which was prepared for this experiment contained 3.78×10^9 organisms per millilitre, and had a total radioactivity of 253,536 counts per minute. The cells had assimilated 25.2% of the available P^{32} in the medium during incubation for 24 hours at 30° C.

The counts taken during migration of the organisms through the core are shown in TABLE IX. Moisture first appeared at the top of the core after thirteen hours, but no appreciable activity could be detected at the top until 44 hours had elapsed. After 44 hours the core was removed from the lucite holder and was split. One half of the core was then coated with the nutrient media and incubated at 30° C. The other half was autoclaved, and an autoradiogram prepared.

Plate III shows the growth of the cells on the split surface of the core after 24 hours incubation; Plate IV shows the growth after 48 hours incubation. Plate V is the autoradiogram from Core #1; it should be noted that the exposure time was only 2 weeks, whereas the autoradiograms for the remaining cores were exposed for 4 weeks in order to obtain better contrast.

TABLE IX

MIGRATION OF P^{32} LABELLED S. MARCESCENS IN BERE A SANDSTONE

Time Lapse	Port Location	Counts per minute in excess of background			
		0°	90°	180°	270°
0 Hours	2"	0	0	0	0
4 Hours	2"	10	15	0	0
	3"	0	0	0	0
8 Hours	2"	9	17	0	0
	3"	0	0	0	0
12 Hours	2"	0	23	0	0
	3"	0	0	0	0
16 Hours	2"	0	23	44	0
	3"	0	11	0	0
	4"	0	0	0	0
20 Hours	2"	29	51	39	37
	3"	18	10	9	41
	4"	18	10	27	13
	5"	0	0	0	21
	6"	0	0	0	0
24 Hours	2"	50	73	36	26
	3"	0	22	13	27
	4"	0	11	5	20
	5"	0	0	0	0
	6"	8	37	9	9
	Top	18	0	23	15
28 Hours	2"	62	103	107	78
	3"	14	36	55	25
	4"	27	0	44	76
	5"	0	5	0	0
	6"	72	0	0	0
	Top	5	4	0	0
36 Hours	2"	67	73	69	22
	3"	0	22	33	4
	4"	0	0	0	0
	5"	0	5	0	0
	6"	0	0	0	0
	Top	0	6	5	6

(continued on page 38)

TABLE IX (continued from page 37)

MIGRATION OF P^{32} LABELLED S. MARCESCENS IN BEREA SANDSTONE

Time Lapse	Port Location	Counts per minute in excess of background			
		0°	90°	180°	270°
40 Hours	2"	42	78	64	34
	3"	14	14	0	8
	4"	0	0	0	0
	5"	0	0	0	0
	6"	0	8	0	0
	Top	11	20	15	18
44 Hours	2"	69	112	96	37
	3"	16	23	17	4
	4"	0	0	0	0
	5"	0	0	0	5
	6"	0	0	21	4
	Top	30	26	8	11

PLATE III

GROWTH OF S. MARCESCENS ON THE SPLIT SURFACE OF CORE #1 AFTER
24 HOURS INCUBATION AT 30°C.



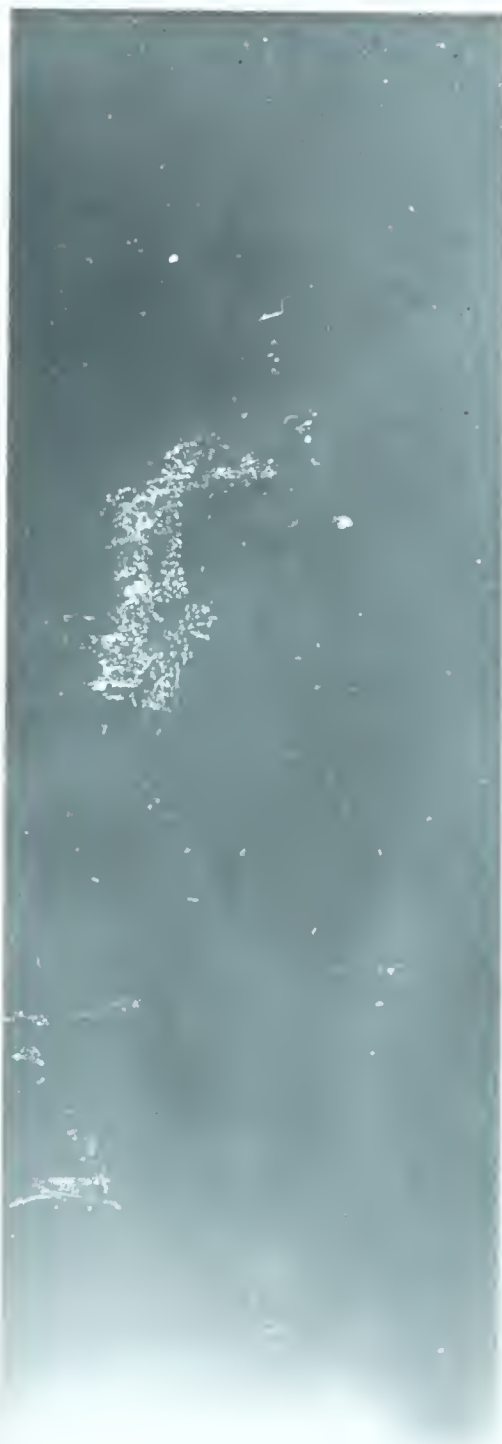
PLATE IV

GROWTH OF S. MARCESCENS ON THE SPLIT SURFACE OF CORE #1 AFTER
48 HOURS INCUBATION AT 30° C.



PLATE V

AN AUTORADIOGRAM OF THE DISTRIBUTION OF P^{32} ON THE SPLIT SURFACE
OF CORE #1



At the end of 44 hours there were 64 millilitres of slurry remaining, which had a total activity of 183,235 c/m. (corrected to zero time). Using the figure for the total count for the slurry at the beginning and at the end of each experiment, the percentage of cells entering the core can be calculated.

$$\frac{183,235}{253,536} \times 100\% = 72.54\%$$

72.54% of the cells were in the slurry at the end of the experiment therefore 27.46% of the labelled cells in the original slurry entered the core.

Core #2 was exactly ten inches long. The slurry which was prepared for this experiment contained 4.04×10^9 organisms per millilitre, and had a total activity of 187,100 c/m. The cells had assimilated 25.7% of the available P^{32} in the medium during incubation for 24 hours at 30° C.

The counts taken during the migration of the organisms through the core are shown in TABLE X. Moisture appeared at the top of the core after 19 hours but no appreciable activity could be detected at the top until 36 hours had elapsed. Migration was complete after 71 hours; the core was removed from the lucite holder and split. Plate VI shows the growth and distribution of S. marcescens on the split surface of Core #2 after 24 hours incubation at 30° C. Plate VII shows the distribution of the P^{32} on the split surface of Core #2. It can be seen that there is a very heavy concentration of P^{32} through the centre of the core. The results presented in TABLE X show that radioactivity could not be detected, by counting at the surface of the core above the

4 inch level whereas appreciable counts were obtained at the top. Thus it is evident that counts cannot be obtained from the labelled organisms which migrate through the centre of the core. The white area at the bottom and along both sides is due to the entrance of light into the container during exposure.

At the time of completion of the experiment there were 27 millilitres of the slurry remaining, which had a total activity of 102,006 c/m.

$$\frac{102,006}{187,100} \times 100 = 54\%$$

54% of the cells were in the slurry at the end of the experiment therefore 46% of the labelled cells in the original slurry entered the core.

Core #3 was exactly 8.25 inches long. The slurry which was prepared for this experiment contained 3.96×10^9 organisms per millilitre and had a total radioactivity of 309,500 c/m. The cells had assimilated 30.2% of the available P^{32} in the medium during incubation for twenty-four hours at 30°C.

The counts taken during migration of the organisms through the core are shown in TABLE XI. Moisture first appeared at the top of the core after twelve hours, but no appreciable radioactivity could be detected at the top until 32 hours had elapsed. Migration was complete after 46 hours; the core was removed from the lucite holder and was split. Plate VIII shows the growth and distribution of S. marcescens on the split surface of Core #3 after twenty-four hours incubation at 30° C. Plate IX shows the distribution of the P^{32} on

TABLE X

MIGRATION OF P^{32} LABELLED S. MARCESCENS IN BEREASANDSTONE

Time Lapse	Port Location	Counts per minute in excess of background			
		0°	90°	180°	270°
0 Hours	2"	0	0	0	0
4 Hours	2"	78	89	56	6
	3"	100	115	22	0
	4"	86	50	0	0
	5"	40	15	0	0
	6"	32	0	0	0
	7"	0	0	0	0
8 Hours	2"	124	88	35	17
	3"	107	80	5	30
	4"	96	56	25	24
	5"	48	21	16	0
	6"	16	8	0	0
	7"	25	0	0	0
	8"	0	0	0	0
12 Hours	2"	79	65	47	33
	3"	73	56	17	0
	4"	44	32	0	0
	5"	20	28	0	0
	6"	13	22	0	0
	7"	6	31	0	0
	8"	13	7	0	0
	9"	0	0	0	0
19 Hours	2"	71	35	64	23
	3"	38	19	18	10
	4"	30	6	0	0
	5"	15	25	0	0
	6"	14	0	0	0
	7"	0	0	0	0
24 Hours	2"	37	22	58	31
	3"	41	25	17	0
	4"	20	24	13	0
	5"	18	24	0	0
	6"	0	0	0	0
	7"	0	0	0	0
28 Hours	2"	755	102	117	40
	3"	52	35	26	15
	4"	31	48	0	0
	5"	18	17	0	0
	6"	9	12	0	0
	7"	13	0	19	0
	8"	0	0	0	0

TABLE X (continued)

Time Lapse	Port Location	Counts per minute in excess of background			
		0°	90°	180°	270°
32 Hours	2"	70	82	99	27
	3"	31	31	15	11
	4"	12	36	5	0
	5"	15	20	0	8
	6"	0	8	5	0
	7"	7	15	0	9
	8"	0	0	0	0
36 Hours	2"	107	47	69	25
	3"	8	14	13	0
	4"	9	18	0	0
	5"	0	0	0	0
	Top	11	14	11	13
46 Hours	2"	44	80	123	58
	3"	5	16	25	18
	4"	5	21	26	6
	5"	11	15	13	0
	6"	0	0	6	0
	7"	19	0	6	0
	8"	13	0	0	0
	9"	11	7	13	0
	Top	22	19	25	20
56 Hours	2"	35	70	106	47
	3"	0	28	24	19
	4"	9	11	17	0
	5"	0	0	7	0
	6"	0	0	8	0
	7"	8	14	6	24
	8"	40	35	10	15
	9"	39	20	29	10
	Top	48	20	24	13
60 Hours	2"	28	70	127	17
	3"	7	13	27	10
	4"	8	20	10	11
	5"	0	16	12	0
	6"	19	11	11	0
	7"	19	11	13	0
	8"	0	0	0	0
	9"	0	6	7	0
	Top	32 c/m. at centre of the core			
71 Hours	2"	45	84	118	50
	3"	20	10	43	35
	4"	0	0	0	17
	5"	25	5	18	0
	6"	0	21	0	7
	7"	25	0	0	27
	8"	6	0	0	22
	9"	18	0	0	47
	Top	21	8	5	30

PLATE VI

GROWTH OF S. MARCESCENS ON THE SPLIT SURFACE OF CORE #2
AFTER 24 HOURS INCUBATION AT 30° C.



PLATE VII

AN AUTORADIOGRAM OF THE DISTRIBUTION OF P^{32} ON THE SPLIT SURFACE OF CORE #2



the split surface of Core #3. The artifact in the upper left hand corner of Plate IX is a drop of epoxy resin which fell on the negative.

On completion of the experiment there were 48.5 millilitres of slurry remaining, which had a total activity of 136,200 c/m.

$$\frac{136,300}{309,500} \times 100 = 44.19\%$$

44.19% of the cells were in the slurry at the end of the experiment therefore 55.81% of the labelled cells in the original slurry entered the core.

Core #4 was exactly 14.25 inches long. The slurry which was prepared for this experiment contained 3.84×10^9 organisms per millilitre and had a total radioactivity of 280,953 c/m. The cells had assimilated 28.22% of the available P^{32} in the medium during incubation for 24 hours at 30° C.

The counts taken during the migration of the organisms through the core are shown in TABLE XII. Moisture first appeared at the top of the core after 47 hours, but no appreciable activity could be detected at the top until 84 hours had elapsed, at which time the core was removed from the lucite holder and split. Plate X shows the growth and distribution of S. marcescens on the split surface of Core #4. Plate XI shows the growth of S. marcescens throughout the cross-section of the core.

Due to the length of Core #4, two pieces of x-ray film had to be used for the autoradiogram. The core slipped off the film and an autoradiogram was not obtained.

TABLE XI

MIGRATION OF P^{32} LABELLED S. MARCESCENS IN BEREASANDSTONE

Counts per minute in excess of background

Time Lapse	Port Location	0°	90°	180°	270°
0 Hours	2"	0	0	0	0
4 Hours	2"	8	15	10	0
	3"	0	0	0	0
8 Hours	2"	17	38	37	36
	3"	14	15	0	11
	4"	14	9	0	0
	5"	0	0	0	0
12 Hours	2"	0	32	12	0
	3"	0	0	0	0
16 Hours	2"	34	23	29	45
	3"	10	0	0	11
	4"	0	0	0	0
20 Hours	2"	41	44	64	37
	3"	0	15	6	9
	4"	0	0	0	0
24 Hours	2"	69	60	54	77
	3"	0	0	0	10
	4"	6	0	0	0
	5"	0	0	0	0
28 Hours	2"	32	41	29	28
	3"	0	15	0	8
	4"	0	12	0	0
	5"	0	0	0	0
32 Hours	2"	72	46	63	87
	3"	13	7	0	12
	4"	10	0	8	6
	5"	7	0	0	9
	6"	0	0	0	0
Top	36 c/m. at the centre of the core				

(continued on page 50)

TABLE XI (continued from page 49)

MIGRATION OF P^{32} LABELLED S. MARCESCENS IN BERE A SANDSTONE

<u>Counts per minute in excess of background</u>					
Time Lapse	Port Location	0°	90°	180°	270°
36 Hours	2"	48	49	46	79
	3"	5	10	20	0
	4"	0	0	0	0
	Top	51 c/m. at the centre of the core			
40 Hours	2"	60	36	34	65
	3"	17	28	19	0
	4"	18	8	8	0
	5"	0	0	0	0
	6"	0	7	6	0
	7"	0	0	0	0
46 Hours	2"	57	51	42	117
	3"	34	28	33	34
	4"	16	18	8	10
	5"	5	0	13	7
	6"	0	0	6	0
	7"	0	0	0	0
	Top	58 c/m. at the centre of the core			

Table 1. Summary of the data collected during the field study.				Date	
Location	Time	Temperature (°C)	Humidity (%)	Wind speed (m/s)	Wind direction
1	08:00	25.0	65.0	1.5	SE
2	09:00	26.5	60.0	2.0	SE
3	10:00	28.0	55.0	2.5	SE
4	11:00	29.5	50.0	3.0	SE
5	12:00	31.0	45.0	3.5	SE
6	13:00	32.5	40.0	4.0	SE
7	14:00	34.0	35.0	4.5	SE
8	15:00	35.5	30.0	5.0	SE
9	16:00	37.0	25.0	5.5	SE
10	17:00	38.5	20.0	6.0	SE
11	18:00	40.0	15.0	6.5	SE
12	19:00	41.5	10.0	7.0	SE
13	20:00	43.0	5.0	7.5	SE
14	21:00	44.5	0.0	8.0	SE
15	22:00	46.0	0.0	8.5	SE
16	23:00	47.5	0.0	9.0	SE
17	00:00	49.0	0.0	9.5	SE
18	01:00	50.5	0.0	10.0	SE
19	02:00	52.0	0.0	10.5	SE
20	03:00	53.5	0.0	11.0	SE
21	04:00	55.0	0.0	11.5	SE
22	05:00	56.5	0.0	12.0	SE
23	06:00	58.0	0.0	12.5	SE
24	07:00	59.5	0.0	13.0	SE
25	08:00	61.0	0.0	13.5	SE
26	09:00	62.5	0.0	14.0	SE
27	10:00	64.0	0.0	14.5	SE
28	11:00	65.5	0.0	15.0	SE
29	12:00	67.0	0.0	15.5	SE
30	13:00	68.5	0.0	16.0	SE
31	14:00	70.0	0.0	16.5	SE
32	15:00	71.5	0.0	17.0	SE
33	16:00	73.0	0.0	17.5	SE
34	17:00	74.5	0.0	18.0	SE
35	18:00	76.0	0.0	18.5	SE
36	19:00	77.5	0.0	19.0	SE
37	20:00	79.0	0.0	19.5	SE
38	21:00	80.5	0.0	20.0	SE
39	22:00	82.0	0.0	20.5	SE
40	23:00	83.5	0.0	21.0	SE
41	00:00	85.0	0.0	21.5	SE
42	01:00	86.5	0.0	22.0	SE
43	02:00	88.0	0.0	22.5	SE
44	03:00	89.5	0.0	23.0	SE
45	04:00	91.0	0.0	23.5	SE
46	05:00	92.5	0.0	24.0	SE
47	06:00	94.0	0.0	24.5	SE
48	07:00	95.5	0.0	25.0	SE
49	08:00	97.0	0.0	25.5	SE
50	09:00	98.5	0.0	26.0	SE
51	10:00	100.0	0.0	26.5	SE
52	11:00	101.5	0.0	27.0	SE
53	12:00	103.0	0.0	27.5	SE
54	13:00	104.5	0.0	28.0	SE
55	14:00	106.0	0.0	28.5	SE
56	15:00	107.5	0.0	29.0	SE
57	16:00	109.0	0.0	29.5	SE
58	17:00	110.5	0.0	30.0	SE
59	18:00	112.0	0.0	30.5	SE
60	19:00	113.5	0.0	31.0	SE
61	20:00	115.0	0.0	31.5	SE
62	21:00	116.5	0.0	32.0	SE
63	22:00	118.0	0.0	32.5	SE
64	23:00	119.5	0.0	33.0	SE
65	00:00	121.0	0.0	33.5	SE
66	01:00	122.5	0.0	34.0	SE
67	02:00	124.0	0.0	34.5	SE
68	03:00	125.5	0.0	35.0	SE
69	04:00	127.0	0.0	35.5	SE
70	05:00	128.5	0.0	36.0	SE
71	06:00	130.0	0.0	36.5	SE
72	07:00	131.5	0.0	37.0	SE
73	08:00	133.0	0.0	37.5	SE
74	09:00	134.5	0.0	38.0	SE
75	10:00	136.0	0.0	38.5	SE
76	11:00	137.5	0.0	39.0	SE
77	12:00	139.0	0.0	39.5	SE
78	13:00	140.5	0.0	40.0	SE
79	14:00	142.0	0.0	40.5	SE
80	15:00	143.5	0.0	41.0	SE
81	16:00	145.0	0.0	41.5	SE
82	17:00	146.5	0.0	42.0	SE
83	18:00	148.0	0.0	42.5	SE
84	19:00	149.5	0.0	43.0	SE
85	20:00	151.0	0.0	43.5	SE
86	21:00	152.5	0.0	44.0	SE
87	22:00	154.0	0.0	44.5	SE
88	23:00	155.5	0.0	45.0	SE
89	00:00	157.0	0.0	45.5	SE
90	01:00	158.5	0.0	46.0	SE
91	02:00	160.0	0.0	46.5	SE
92	03:00	161.5	0.0	47.0	SE
93	04:00	163.0	0.0	47.5	SE
94	05:00	164.5	0.0	48.0	SE
95	06:00	166.0	0.0	48.5	SE
96	07:00	167.5	0.0	49.0	SE
97	08:00	169.0	0.0	49.5	SE
98	09:00	170.5	0.0	50.0	SE
99	10:00	172.0	0.0	50.5	SE
100	11:00	173.5	0.0	51.0	SE

PLATE VIII

GROWTH OF S. MARCESCENS ON THE SPLIT SURFACE OF CORE #3
AFTER 24 HOURS INCUBATION AT 30° C.

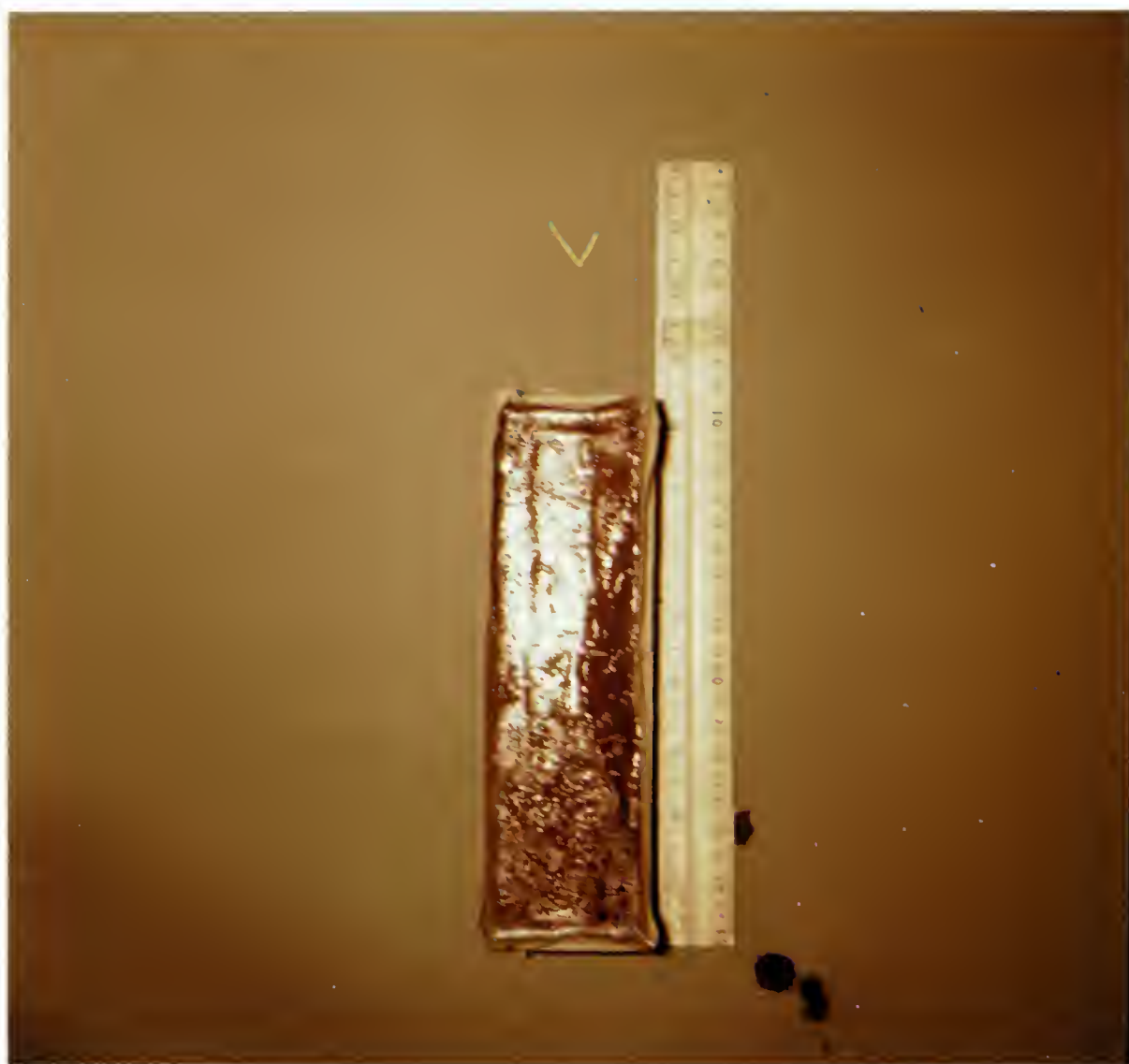


PLATE IX

AN AUTORADIOGRAM OF THE DISTRIBUTION OF P^{32} ON THE SPLIT SURFACE OF CORE #3



TABLE XII

MIGRATION OF P^{32} LABELLED S. MARCESCENS IN BEREASANDSTONE

Time Lapse	Port Location	Counts per minute in excess of background			
		0°	90°	180°	270°
0 Hours	2"	0	0	0	0
4 Hours	2"	18	24	15	36
	3"	17	8	0	16
	4"	7	0	0	0
	5"	0	0	0	0
8 Hours	2"	24	40	13	36
	3"	0	8	12	17
	4"	0	13	0	6
	5"	0	0	0	0
12 Hours	2"	25	45	30	80
	3"	21	18	17	12
	4"	0	5	18	0
	5"	0	0	0	0
20 Hours	2"	30	52	63	70
	3"	6	13	16	11
	4"	7	0	10	7
	5"	10	6	16	10
	6"	0	0	0	0
24 Hours	2"	53	64	70	110
	3"	16	15	19	16
	4"	6	0	16	8
	5"	13	0	0	0
	6"	7	0	0	0
	7"	0	0	0	0
28 Hours	2"	64	76	85	79
	3"	18	14	26	13
	4"	0	14	14	6
	5"	0	11	6	20
	6"	10	0	0	0
	7"	0	0	0	0
32 Hours	2"	83	78	93	101
	3"	16	20	8	18
	4"	0	0	0	0
	5"	0	0	0	0
	6"	16	0	0	0
	7"	0	0	0	0

(continued on page 54)

TABLE XII (continued from page 53)

MIGRATION OF P³² LABELLED S. MARCESCENS IN BERE A SANDSTONE

Time Lapse	Port Location	Counts per minute in excess of background			
		0°	90°	180°	270°
36 Hours	2"	97	70	97	106
	3"	6	0	15	13
	4"	0	0	11	11
	5"	5	0	0	0
	6"	0	0	0	0
44 Hours	2"	88	121	167	130
	3"	15	16	32	16
	4"	0	0	0	22
	5"	13	0	0	0
	6"	0	0	9	0
	7"	0	0	0	0
48 Hours	2"	77	74	140	131
	3"	28	10	12	34
	4"	0	0	0	0
	5"	0	0	0	0
52 Hours	2"	62	72	92	113
	3"	14	10	10	21
	4"	0	0	7	5
	5"	0	0	0	0
56 Hours	2"	57	81	87	125
	3"	16	26	9	14
	4"	17	23	11	15
	5"	11	16	8	9
	6"	13	10	0	0
	7"	13	7	0	0
	8"	7	7	0	0
	9"	0	0	0	0
60 Hours	2"	107	98	162	106
	3"	52	41	66	58
	4"	24	13	6	10
	5"	12	12	7	5
	6"	6	12	7	0
	7"	0	14	0	7
	8"	0	17	0	14
	9"	0	0	0	0

(continued on page 55)

TABLE XII (continued from page 54)

MIGRATION OF P^{32} LABELLED S. MARCESCENS IN BEREASANDSTONE

Counts per minute in excess of background					
Time Lapse	Port Location	0°	90°	180°	270°
68 Hours	2"	140	201	184	97
	3"	93	86	168	65
	4"	73	6	68	45
	5"	50	0	33	21
	6"	36	0	11	5
	7"	0	9	0	5
	8"	0	0	0	0
72 Hours	2"	75	77	129	106
	3"	18	12	31	31
	4"	7	11	17	6
	5"	0	0	0	9
	6"	0	0	0	11
	7"	0	0	0	0
	8"	0	13	0	17
	9"	0	8	0	0
	10"	7	10	0	0
	11"	0	9	0	0
	12"	0	0	0	0
76 Hours	2"	65	109	104	107
	3"	59	28	41	6
	4"	11	9	18	0
	5"	0	5	0	0
	6"	0	26	0	9
	7"	0	0	7	7
	8"	0	5	0	10
	9"	0	0	0	0
80 Hours	2"	171	187	255	200
	3"	110	78	72	35
	4"	25	23	35	42
	5"	0	0	0	10
	6"	13	0	0	0
	7"	0	0	0	0
	8"	0	0	0	0
84 Hours	2"	182	176	248	196
	3"	45	36	34	78
	4"	17	33	13	19
	5"	0	0	0	0
	6"	0	0	14	0
	7"	0	0	0	0
	Top	62 c/m. at the centre of the core			

PLATE X

GROWTH OF S. MARCESCENS ON THE SPLIT SURFACE OF CORE # 4
AFTER 24 HOURS INCUBATION AT 30° C.

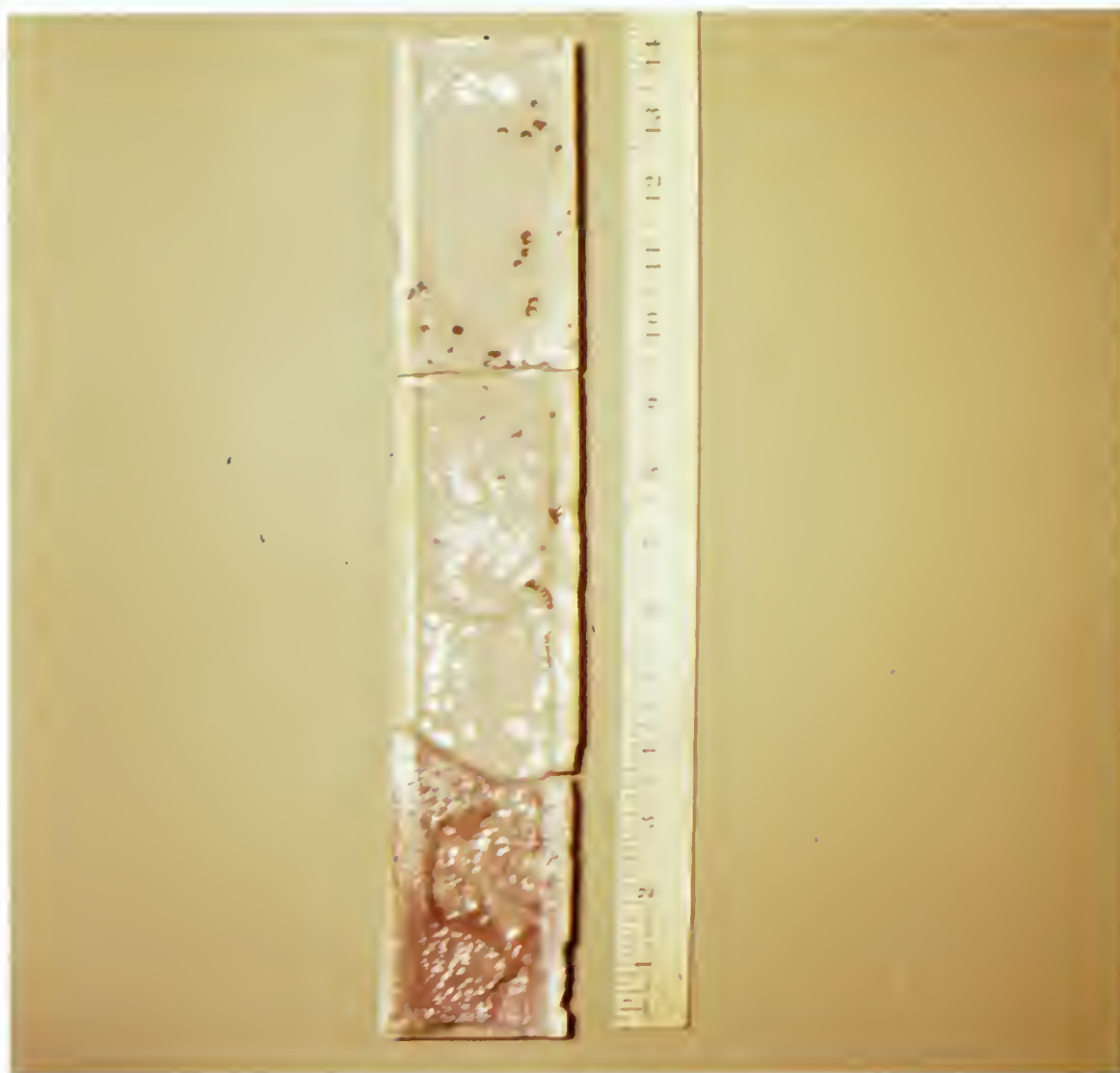


PLATE XI

GROWTH OF S. MARCESCENS THROUGHOUT THE
CROSS-SECTION OF CORE # 4



On completion of the experiment there were 21 millilitres of slurry remaining which had a total activity of 89,334 c/m.

$$\frac{89,340}{280,954} \times 100 = 31.8\%$$

31.8% of the cells were in the slurry at the end of the experiment therefore 68.2% of the labelled cells in the original slurry entered the core.

DISCUSSION

The results presented in TABLES IX, X, XI and XII show that the movement of the organisms through the sandstone is very random. As the exact porosity of these cores was not known it was impossible to correlate the rate of migration to the pore size of the cores.

In all experiments the labelled organisms were detected at the two inch level four hours after the experiment was started. Initially the suspension is rapidly drawn into the core due to the negative pressure produced within the cores during the drying process. * There is a quite noticeable delay before the organisms can be detected at a higher level. This is probably due to plugging which follows the initial rapid migration. The majority of the pore are believed to be between one and two microns in diameter, therefore the organisms may become trapped in these smaller tortuous pores and migration is slowed until the organisms either grow through these fine pores into a larger pore space or manage to orientate themselves in such a way as to slip through the fine pores.

Growth through the fine pores of sandstone has been proven by Woodhead and Wood ⁴³. They reported that Staphylococcus pyogenes aureus could grow through a one inch natural sandstone bacterial filter in 48 hours. The silica particles of this piece of sandstone were reported to be one to three microns in diameter, thereby indicating a very tight formation, with a very small pore diameter.

Sharpley ³³ states that bacteria do not behave in the same manner as inanimate material when filtered through a porous medium.

* see Fekete⁷.

The surface charge and the external structure of the organisms cause an accelerated plugging effect.

The flagella of motile organisms may accelerate plugging in the finer pores due to entanglement, but if a motile organism enters a large pore, its rate of migration will be much greater than that of a non-motile organism³⁹.

Nutrient medium was not used as a vehicle in our migration studies for two reasons. First, the presence of a complete nutritional environment would probably not be found under natural conditions. Secondly, such a medium may induce plugging itself due to protein precipitation by the soluble salts present in the cores.

Excellent correlation between the distribution of the P^{32} and the growth of the cells on the split surface of Core #1 can be seen in Plates III and V. In the subsequent migration studies the same degree of correlation cannot be seen. However, it is interesting to note that in Cores #2 and #3 where there was fairly uniform distribution of the organisms over the length of the core as shown by the surface culture, the autoradiograms show an intense concentration of P^{32} almost exactly through the centre of the cores.

The distribution of the S. marcescens in Core #3 is very even, therefore the pore size throughout the core must be fairly uniform.

CONCLUSIONS

Evidence has been presented which conclusively proves that bacteria can penetrate and migrate through Berea Sandstone. The rationale of the technique for tracing the migration of the organisms is sound. However the technique could be improved by using cores of a smaller diameter, thereby permitting the detection of the radioactivity emanating from the central portion of the core.

It is suggested that oil permeated cores, and cores of known porosity be used in future studies, thus the effect of oil on the migration of microorganisms could be determined and also the rate of migration could be correlated to porosity. It would seem desirable to study the rate of migration of organisms of various sizes and shapes through cores of known porosity.

If future studies indicate that organisms can penetrate and migrate through or into the various types of rock formation, more study will be required to establish that so called fossilized microorganisms found in geological specimens were actually trapped at the time of the rock formation and did not migrate subsequent to the rock formation.

If microorganisms are shown to penetrate and migrate in petroliferous formations, it is feasible that the microorganisms could migrate to a natural petroleum reservoir and subsequently alter the petroleum by their metabolic activities.

APPENDIX

MATERIALS

Bacto Nutrient Broth

Peptone..... 5 Gm.

Beef Extract..... 3 Gm.

Distilled Water, add to 1000 ml.

Glucose..... Fisher Scientific Company

Reagent Grade, anhydrous d-glucose Cat.No. D-16

Celite 281..... Johns Manville Company

Amorphous diatomaceous silica powder

Epon Resin 828..... Shell Chemical Company, a division of
Shell Oil Company, Houston, Texas.

Epon Curing Agent T-1..Shell Chemical Company, a division of
Shell Oil Company, Houston, Texas.

Kodak Medical X-ray Film, Blue Brand,
Canadian Kodak Company, Ltd.,
Toronto, Ontario.

Photographs..... The autoradiograms were developed by
Mr. E. Beaumont of the Provincial Public
Health Laboratory, Edmonton, Alberta.
The coloured photographs were developed
and all prints were processed by Abel's
Photofinishers, Calgary, Alberta.

REAGENTS FOR THE PHOSPHATE DETERMINATION 12

10 N. Sulfuric Acid... 282 cc. of concentrated H_2SO_4 (sp. gr. 1.84)
to which was added 600 mls. of distilled
water, the solution was allowed to cool and
distilled water was added to a final volume
of 1,000 mls.

7.5% Sodium Molybdate..7.5 Gms. sodium molybdate dissolved in 100
mls. of distilled water. Two minims of
toluene were added to prevent mold growth.

Stannous Chloride Solution.... 10 Gms. of stannous chloride dissolved
in 25 mls. of concentrated HCl.
Layer with toluene to prevent
oxidation and store in a brown bottle.

TERMINOLOGY

- 60/120 This refers to the passage of the quartz sand through sieves. The sand will pass through a sieve with 60 openings per square inch but will not pass through a sieve with 120 openings per square inch.
- Specific Activity (S/A).... In this thesis this term is used with two different connotations:
- S/A = $\mu\text{g. P}^{32}/\mu\text{g. total phosphorus}$
= Specific labelled content.
 - S/A = counts per minute per mg.
total phosphorus.
- Microcurie (μc) The amount of a labelled element in which the number of disintegrations per second is 3.7×10^4 .
- Background The level of natural atmospheric radiation in counts per minute. The background varied in our experiments between 34 and 43 c/m.
- Roentgen A unit of radiological dose. That quantity of X or γ radiation such that the associated corpuscular emission per 0.001293 Gm. of air, produces, in air, ions carrying one electrostatic unit of quantity of electricity of either sign.

CALCULATION OF THE PORE SIZE IN BEREASANDSTONE

The pore size was calculated from the Capillary Pressure Curve and Data for Berea Sandstone in Raleigh's²⁸ thesis of pages A-18 and A-19. The radius of the pores were calculated using the formula:¹

$$D_{(ri)} = \frac{P_{ci}}{r_i} \frac{dS_m}{dP_c}$$

P_{ci} = capillary pressure in cm. of Hg.

r_i = pore entry radius

σ = interfacial tension

θ = contact angle

S_m = Mercury saturation, as a percentage of the pore volume.

Capillary pressure was converted from p.s.i. to cm. of Hg.

using the formula:

$$P_{ci} = \frac{P_c \times 76}{14.7}$$

P_c = capillary pressure in p.s.i.

76 = atmospheric pressure in cm. of Hg.

14.7 = specific gravity of Hg.

The bulk volume of the core tested by Raleigh²⁸ was 18.18 cc., the pore volume was 24.5% or 4.454 cc. The fraction of the pore space occupied by Hg. at the various pressure readings was calculated by multiplying the Hg. fraction of the pore volume by the total pore volume, divided by the total bulk volume:

$$S_m = \frac{\text{Hg. fraction of Bulk Volume} \times 4.454}{18.18}$$

The radius of the pores were calculated using the formula:

$$r_i = \frac{2\sigma \cos \theta}{P_{ci}}$$

The slope of the capillary pressure curve was calculated at the various pressure readings using the formula:

$$\text{slope} = \frac{S_m}{P_{ci}}$$

The pore distribution factor ($D_{(ri)}$) was calculated using the formula:

$$D_{(ri)} = \frac{P_{ci}}{r_i} \times \text{slope} = \frac{P_{ci}}{r_i} \frac{dS_m}{dP_c}$$

The distribution factor was then plotted on a graph against the pore radius.

FIGURE VII
PORE SIZE DISTRIBUTION IN BERE A SANDSTONE

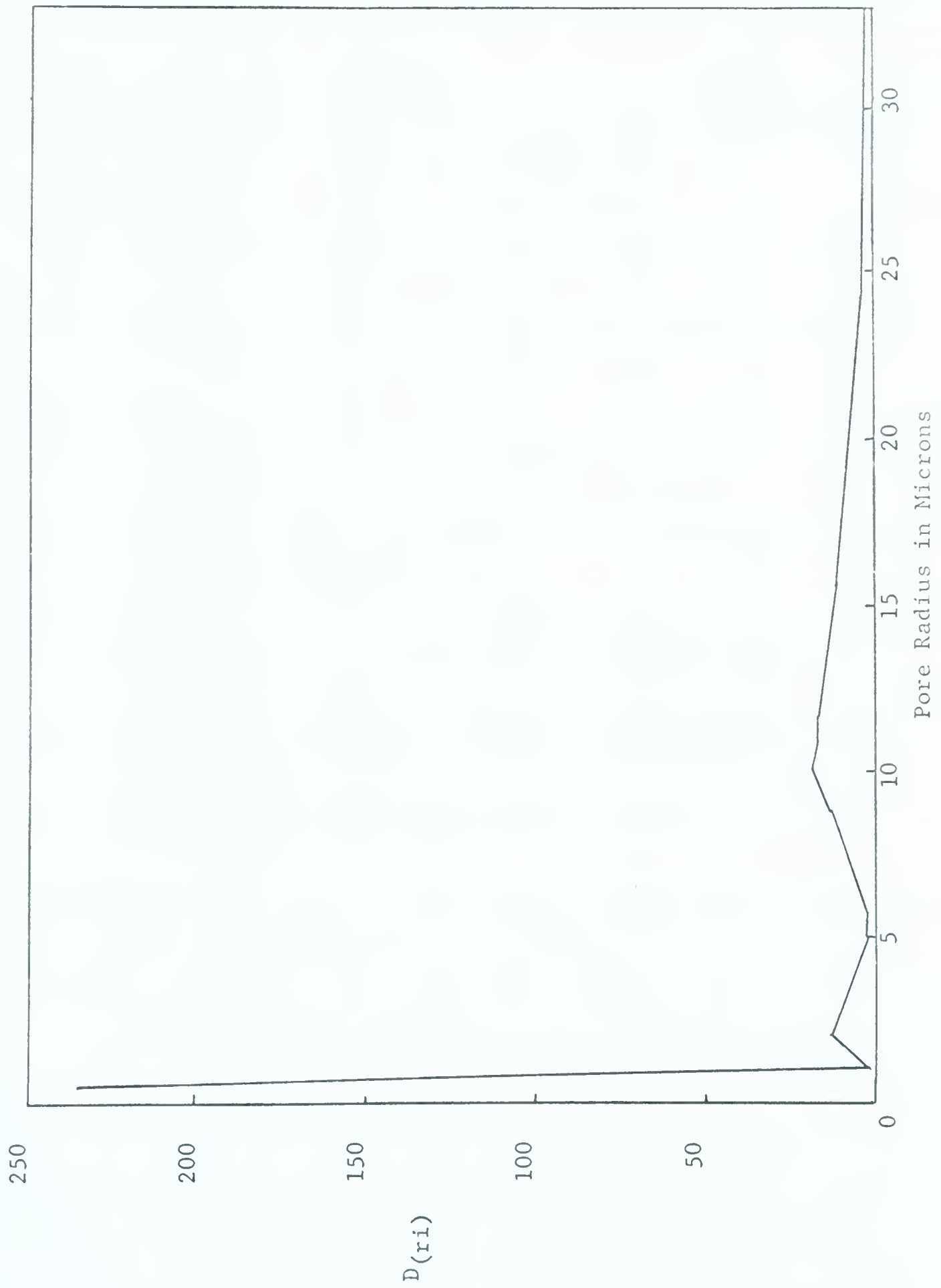


TABLE XIII

CALCULATION OF THE PORE SIZE DISTRIBUTION IN BEREA SANDSTONE

Pc	Pci	Hg. fraction of the bulk volume	Sm	ri	Slope $\frac{Sm}{Pci}$	D (ri)
3.9	20.16	0.008	0.00196	36.47	3.0	1.66
5.7	29.47	0.010	0.00245	24.90	3.0	3.55
8.5	43.94	0.065	0.0160	16.78	3.45	9.05
11.4	58.94	0.126	0.0309	12.48	3.2	15.11
13.6	70.31	0.142	0.0348	10.46	2.45	16.47
17.0	87.89	0.161	0.0394	8.39	1.00	10.45
22.0	113.74	0.165	0.0404	6.46	0.125	2.41
27.0	139.59	0.168	0.0412	5.27	0.0938	2.49
55.0	284.37	0.181	0.0443	2.58	0.1	11.02
108.0	558.36	0.192	0.0470	1.32	0.051	2.16
316.0	1633.74	0.206	0.0505	0.45	0.066	239.62

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